# STUDIES OF PHOTODYNAMIC ACTION

## III. The Difference in Mechanism Between Photodynamic Hemolysis and Hemolysis by Non-irradiated Eosine

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Dyes which bring about photodynamic hemolysis, in many instances, bring about the same effect in the absence of light (Dunkelwirkung), when the dye is present in sufficiently high concentration. This suggests the possibility that a reaction of the dye with cell constituents which is independent of light underlies the hemolysis; and that this reaction is accelerated by the activation of dye molecules by absorbed radiation, with the result that hemolysis occurs in lower concentrations of the dye. Certain rough correlations between the effect of the non-irradiated dyes and the photodynamic effects have been pointed out by Jodlbauer and Haffner (1921*a*) and by Blum (1930*b*) which would support this thesis, but it is possible that hemolysis is initiated by entirely different mechanisms in the two cases. Photodynamic hemolysis has been shown to require the presence of molecular oxygen (Hasselbalch, 1909; Schmidt and Norman, 1922), and there seems little doubt that this phenomenon is dependent upon oxidations by molecular oxygen activated in some way by light. Obviously, if the hemolysis produced by the non-irradiated dye is dependent upon the same reactions, it must likewise be inhibited by the absence of molecular oxygen. The attempt to separate the two processes on this basis has been the object of the following experiments.

### Experimental

Quantitative experimental treatment of this problem meets with various difficulties. Measurement and comparison of the oxygen consumption during hemolysis by irradiated and non-irradiated dyes meets the *a priori* objection that certain dyes greatly alter the normal metabolism of cells without apparent destructive effects (see Barron and Hoffman, 1930), which might result in false conclusions as to the oxygen consumption of the process leading to hemolysis. Reducing the partial pressure of oxygen in any way, with the object of studying the effect on the hemolytic process, brings about changes in hydrogen ion concentra-

tion within the red blood cell due to the formation of reduced hemoglobin. This change in hydrogen ion concentration may considerably affect the hemolytic process (Jodlbauer and Haffner, 1921*b*; Blum, 1930*b*), without reference to oxidative reactions. Such factors offer considerable difficulties in quantitative experimentation, and it has, therefore, appeared wise to attempt only to demonstrate qualitatively, whether or not hemolysis by dyes may occur in the absence of light and oxygen.

The method employed has been as follows: Series of eosine solutions were prepared covering a range of concentrations which included the minimum concentration found to bring about hemolysis in air in the dark. The solutions were made up with isosmotic phosphate buffers usually at pH 7.0, according to the procedure described by Blum (1930*a*). Suspensions of 0.5 per cent red blood cells were made with these solutions, oxygen removed, and one series exposed to sunlight, while the other was maintained in the dark. If hemolysis by the non-irradiated dye, as well as photodynamic hemolysis, requires oxygen, hemolysis should not appear at any dye concentration in either the irradiated or the non-irradiated series. On the other hand, if the action of the non-irradiated dye does not require oxygen, there should be a concentration in each series above which hemolysis should occur. In the latter case the minimum concentration at which hemolysis occurs need not be exactly the same as the corresponding minimum in air, since the removal of oxygen would result in a change in the hydrogen ion concentration within the cell which might cause a shift in this minimum.

The validity of the results obtained in this way depends upon the removal of oxygen to a level which will not allow the oxidative mechanisms leading to hemolysis to proceed at a demonstrable rate. The only criterion for this is the complete inhibition of hemolysis in the irradiated systems. This was found difficult, the difficulty lying apparently in the removal of the oxygen from the cells themselves. The suspensions which we have found convenient for the study of the hemolytic process contain 0.5 per cent red blood cells, and it may be readily calculated that in such a system the cells contain about one-fourth of the total oxygen in the system. The cells contain about forty per cent by volume of oxygen, or 0.2 cc. per 100 cc. of suspension containing 0.5 per cent cells. From the absorption coefficient of oxygen in water approximately three cc. of oxygen are absorbed in 100 cc. of water at 25° C., and since our solutions are saturated with air and not with oxygen, they should contain approximately one-fifth of this quantity or 0.6 cc. per 100 cubic centimeters. Thus there is, roughly, one-third as much loosely-bound oxygen in the cells as there is dissolved oxygen in the surrounding solu-

tion. It is thus apparent that the oxygen must be removed from the cell as well as from the solution in order to establish the desired low oxygen tension. It was not found possible to completely inhibit the effects of the irradiated dye by reducing the atmospheric pressure above the solution. It was likewise found difficult to obtain conclusive results by attempting to remove the oxygen by bubbling nitrogen through the solutions for a considerable time. It was found, however, that definite results could be obtained by using carbon monoxide to remove the oxygen from the cells. The procedure in these cases was first to bubble nitrogen through a series of tubes, each containing 2 cc. of dye solution of a given concentration without cells for 15 to 20 minutes to ensure the removal of oxygen from the solutions. Carbon monoxide was bubbled through a 50 per cent suspension of washed red blood cells to

TABLE I

Irradiated systems exposed to mid-day sunlight for 1 hour (12:15 p.m.–1:15 p.m. August 21, 1931). Observations made at the end of 6 hours following mixing of cells with dye solution. *H* = complete hemolysis; (*H*) = partial hemolysis. Solutions contain sodium phosphate buffer isosmotic with 0.15 M NaCl, pH 7.0, + 0.5 per cent r. b. c. Human.

| Eosin Concentration | *a*. Systems in Air | | *b*. Systems in CO | | *c*. Systems in $\frac{CO}{O_2} = \frac{80}{20}$ | |
|---|---|---|---|---|---|---|
| | Irradiated | Not Irradiated | Irradiated | Not Irradiated | Irradiated | Not Irradiated |
| *per cent* | | | | | | |
| 1.4 | H | H | H | (H) | H | H |
| .7 | H | (H) | H | (H) | H | (H) |
| .35 | (H) | — | — | — | (H) | — |
| .175 | (H) | — | — | — | (H) | — |
| .087 | (H) | — | — | — | (H) | — |
| .044 | H | — | — | — | H | — |

remove the oxygen from these; 0.2 cc. of this suspension was then added to each tube to form a 0.5 per cent suspension of cells, the tubes being opened to the air for as short a time as possible in order to avoid the entrance of oxygen. The suspensions were then flushed out with about 400 cc. of carbon monoxide. Such treatment was found very effective in inhibiting the photo-reaction, but, as will be seen by reference to Table I, did not inhibit the action of the non-irradiated dye. In order to rule out any possible specific effect of carbon monoxide, similar systems were treated with a mixture of 20 per cent oxygen and 80 per cent carbon monoxide.

Table I presents the results of a typical experiment, in which three series of cell suspensions were exposed to sunlight, (*a*) in air, (*b*) in

carbon monoxide, and (*c*) in a mixture of carbon monoxide and oxygen, while three similar series were maintained in the dark. The results show that the photodynamic effects are completely inhibited by the absence of oxygen, while the effects of the non-irradiated dye are not. The fact that the photo-effect is completely inhibited in an atmosphere of carbon monoxide indicates that the oxygen content of the system has been lowered sufficiently so that hemolysis by the non-irradiated dye should also be inhibited if it is dependent upon the same oxidative reactions as the photodynamic effect. The fact that carbon monoxide does not inhibit the light reaction when oxygen is present indicates that the inhibition is not a specific action of the carbon monoxide but is due to lack of oxygen. While the results of such experiments vary somewhat with regard to the rate of development of hemolysis, in no case has it been possible to completely inhibit hemolysis by non-irradiated dyes. A certain amount of the variation may be due to temperature differences. The non-irradiated systems were maintained at a temperature of approximately 25° C. during the period before the observations were made. On the other hand, the irradiated systems were exposed during the one-hour period of the irradiation to a variable temperature, which, however, was never higher than 27° C. and in some experiments was considerably lower than the temperature of the non-irradiated systems. So far as can be determined, by such qualitative observation as we have used, the rate of hemolysis is somewhat decreased in the systems in contact with CO or mixtures of CO and $O_2$. It seems probable that this is due to the difference in hydrogen ion concentration of the cells containing carboxyhemoglobin from that of those containing oxyhemoglobin. It is possible, of course, that in the series exposed to light, the carboxyhemoglobin is dissociated to some extent by the action of light with the formation of oxyhemoglobin or reduced hemoglobin, depending upon whether oxygen is present or not. This might account for some differences in the rate of hemolysis between irradiated and non-irradiated systems.

The fact that hemolysis is completely inhibited in the absence of oxygen excludes the possibility that reactions of the type described by Levaillant (1923) and Windaus and Borgeaud (1928) may bring about the destructive changes leading to hemolysis. These reactions take place in the absence of oxygen and may be considered as oxidations in which the dye acts as a hydrogen acceptor. The dye is reduced in these cases to the colorless leucobase, and the fact that no bleaching of the dye was observed in our experiments indicates that reactions of this type did not occur to any appreciable extent.

While it was not found possible, as mentioned above, to inhibit the photo-effect completely by evacuation or by bubbling nitrogen through the suspensions, it was found that such treatment markedly decreased the photo-effect, but did not alter the hemolytic effect of the non-irradiated dye. Similar results were observed when the attempt was made to inhibit these reactions by the use of reducing agents in the solution. Experiments were carried out using $Na_2SO_3$, $Na_2S_2O_3$, and $NaNO_3$ in concentrations up to 0.1M. It was found impossible to completely inhibit the photo-reaction by means of these reducing agents, and therefore it cannot be assumed that the reducing power of the solution is sufficient to completely inhibit the action of the non-irradiated dye if it involves the same process as the photo-reaction. However, the fact that these reagents have no apparent tendency to inhibit the dark reaction indicates that the two processes are essentially different. Thus, while the above evidence may not, perhaps, be considered as absolutely conclusive, collectively it gives strong support to the view that the dark reaction is not an oxidation by molecular oxygen, whereas the photo-reaction is.

## Discussion

Hasselbalch (1909) performed experiments to test whether the hemolysis by non-irradiated dyes requires oxygen, and reported results contrary to those described above. Using red blood cells in suspension in isotonic NaCl solution which contained a given concentration of dye sufficient to bring about hemolysis in the absence of light, he found that the evacuation of the air above the solutions inhibited the hemolysis. Evacuation under these conditions would remove $CO_2$ as well as $O_2$, and since the solutions in which the cells were suspended were unbuffered, the removal of $CO_2$ must have decreased their hydrogen ion concentration. Furthermore, the removal of both $CO_2$ and $O_2$ from the cells themselves must have resulted in a decrease of hydrogen ion concentration within the cells due to formation of reduced hemoglobin. Hemolysis by non-irradiated fluorescein dyes is markedly affected by hydrogen ion concentration (Jodlbauer and Haffner, 1921*b*; Blum, 1930*a*), the minimum concentration necessary to bring about hemolysis increasing as the hydrogen ion concentration decreases. Thus it seems quite probable that the results obtained by Hasselbalch with fluorescein dyes (eosine and rose bengal) were due to the decrease of hydrogen ion concentration to a value at which the concentration of the dye employed would not produce hemolysis in the absence of light. The justification of this criticism will appear upon the examination of the tables given

by Blum (1930*b*).[1] Hasselbalch also used quinine hydrochloride and quinine bisulphate, finding that evacuation prevented hemolysis in the former but not in the latter case. This variation in effect indicates that the factor affecting hemolysis was probably something other than the $O_2$ content of the system. Although, as stated above, changes of hydrogen ion concentration must have occurred in our systems due to the formation of carboxyhemoglobin, this did not mask the effect of $O_2$ lack because the observations were made over a wide range of dye concentrations.

## Experimental—The Effect of Cyanide on Photodynamic Hemolysis

It has been suggested that the mechanism of photodynamic action involves the normal respiratory mechanisms of the cells themselves (*e.g.*, Metzner, 1919 and 1921). If this were true, photodynamic hemolysis should be inhibited by the inhibition of the respiratory enzymes. In the above experiments it was found that the photodynamic effects are not inhibited by CO provided $O_2$ is present. The presence of CO should partially inhibit all the respiratory oxidative mechanisms of the cell except the aerobic dehydrases.[2] However, light decreases the inhibitory effect of CO on certain of these mechanisms ("respiratory enzyme" of Warburg, "indophenol oxidase" of Keilin), and it is possible that the inhibitory effect of CO was very slight in the systems where mixtures of CO and $O_2$ were used (Warburg, 1926).

To test this question further, cyanide was used to inhibit respiratory enzymes. Series of dilutions of eosine were prepared as above, to a part of which M/100 KCN was added. Red blood cells were added (0.5 per cent) and a part of the KCN series was exposed to light together with control series not containing KCN; other KCN and control series were maintained in the dark. In no case could a difference be detected between the KCN series and the controls in either the irradiated or the non-irradiated systems. These experiments are in agreement with those of Loeb (1907) and Moore (1928), who found that KCN did not inhibit destructive changes in echinoderm eggs by eosine and sunlight, and of Baumberger et al. (1929), who found that cyanide did not inhibit the photodynamic action of methylene blue in preventing the clotting of blood plasma. Cooke and Loeb (1909) found that KCN

[1] The values for molar concentrations of dye given in these tables are in error; the decimal point should in all cases be moved one place to the right. Hasselbalch used M/200 eosine in his experiments, and it will be seen that this concentration is, according to these tables, one at which a small difference in hydrogen ion concentration might determine the occurrence or non-occurrence of hemolysis.

[2] The nomenclature here used is that of Dixon (1929).

increased the photodynamic effects of some dyes on eggs, but this may have been due to hydrogen ion concentration effects.

The addition of M 100 KCN should serve to inhibit markedly all the known respiratory mechanisms with the exception of the aërobic dehydrases, including those in which light interferes with inhibition by carbon monoxide. If these mechanisms played a part in the production of photodynamic hemolysis, the inhibitory effect of the cyanide should be reflected in a reduction of hemolysis. As stated above, no such decrease could be observed.

While it is possible that the aërobic dehydrases may play a part, it seems probable that the photodynamic effects are the result of direct oxidation of cell constituents by molecular oxygen, the activation of the $O_2$ resulting from light energy absorbed by a sensitizer and completely independent of activation by cellular enzymes. The destruction of respiratory enzymes might play a more important part in cells in which respiration is more active than in red blood cells, and may possibly account for the induced tropisms of Metzner (1919, 1921), as he suggests, but this explanation has no experimental support.

These experiments also suggest that hydrogen peroxide is not formed as an intermediate step in photodynamic action. If $H_2O_2$ took a part in the oxidations, catalase should tend to oppose the photodynamic effect by its destruction; in such a case the inhibition of catalase by cyanide should result in increased photodynamic effects. As stated above, cyanide has no effect whatsoever on photodynamic hemolysis, and since this is true, it appears improbable that $H_2O_2$ is formed as an intermediate. This does not, however, deny the formation of intermediate organic peroxides which would not be attacked by catalase.

## Summary

1. The absence of molecular oxygen completely inhibits photodynamic hemolysis but does not inhibit the hemolytic action of the non-irradiated dye. The two phenomena are thus dependent upon different fundamental mechanisms.

2. Cyanide does not inhibit hemolysis either by the irradiated or non-irradiated dye. Thus the respiratory mechanisms of the cell, with the exception of the aërobic dehydrases, cannot play a part in the production of photodynamic hemolysis.

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